Developing an siRNA Therapy to Fight Fatty Liver Disease

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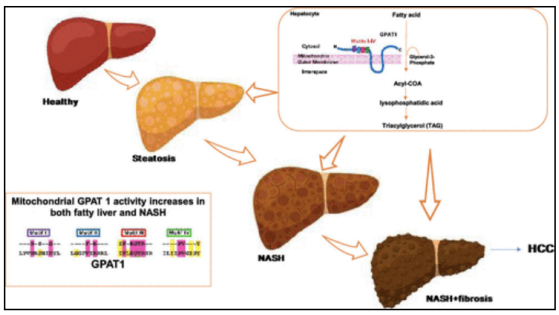
Non-alcoholic fatty liver disease (NAFLD) is more common than one might expect, affecting about 25% of the global population. Those with preexisting conditions, such as obesity, metabolic syndrome, or Type 2 Diabetes are much more likely to develop NAFLD though it can develop in anyone (Maurice *et al.*, 2018). NAFLD is a spectrum disease consisting of stages: the first stage is fatty liver, also known as steatosis. This is when excessive fat (or triglyceride) builds up in the hepatocytes. Diet and exercise can typically help reduce liver fat at this stage. The second stage is nonalcoholic steatohepatitis, or NASH. This is when the excessive fat starts to cause cell bloating, cell degeneration, and infiltration of inflammation cells into the liver. This is the stage when most people are diagnosed. It is also the stage where a tipping effect can occur where it is hard to reverse the disease state. Some individuals with NASH will progress to a more serious stage where collagen builds up in the liver, causing fibrosis. This stage is called "cirrhosis." Individuals with cirrhotic livers typically need a liver transplant to survive. It has also been shown that diabetes is a major factor in one's progression from NAFLD to cirrhosis; those with diabetes were seen to develop cirrhosis almost three times more frequently than those without according to the study performed by Porepa et al (Li *et al.*, 2018). A small subset of individuals with NASH may progress to hepatocellular carcinoma (HCC).

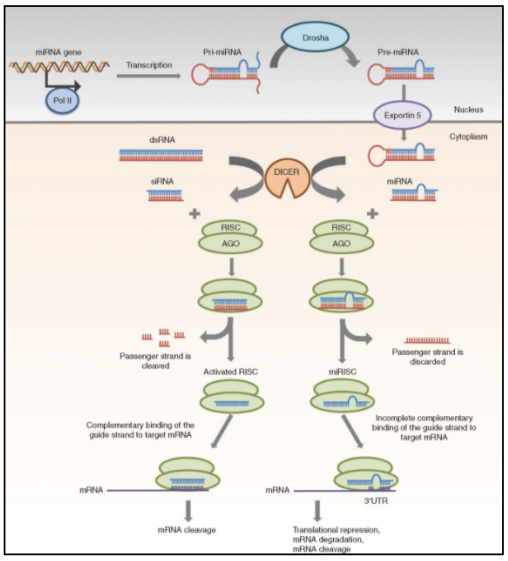
Currently it is estimated that about 80 million adults in the United States fall somewhere on the NAFLD spectrum; 16 million of these individuals are predicted to have NASH. At this time there are at least 70 programs being evaluated to treat NAFLD, however there are still no approved therapies for NASH/cirrhosis. A newer way of finding therapeutic targets for diseases like NASH is to start at the level of human genetic validation. Genome Wide Association Studies (GWAS), for example, led to the identification of single nucleotide polymorphisms (SNPs) in genes that associate with disease phenotypes. In the case of NAFLD, this may be a SNP that alters normal triglyceride (fat) metabolism in the liver. Recently an international group of researchers used the United Kingdom Bio Bank (UKBB) database to search for genetic mutations that associate with NAFLD-related disease phenotypes. The authors identified mutation in glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) associated with increased alanine transaminase levels, a non-invasive measure of liver disease. Carriers of this mutation also exhibit increased triglyceride levels in their livers, increased serum lipid levels and increased incidence of cirrhosis. This particular mutation in GPAM (called rs272951) is a SNP missense mutation resulting in the substitution of isoleucine at position 43 to



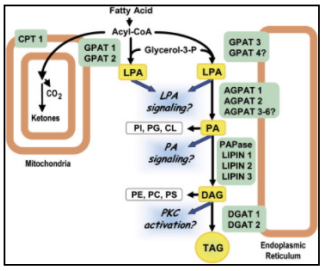
Liao *et al.*, 2020

valine. The minor allele frequency of this mutant allele in the general population is approximately 26%, thus it is a common mutation.

Researchers discovered that when GPAT1 was overexpressed in hepatocytes, it resulted in the exogenous fat being directed towards triacylglycerol (TAG), rather than beta-oxidation, resulting in significant fatty liver. When fed an obesity-inducing diet, GPAM-deficient mice were protected from fatty liver and elevated serum lipid levels (Hammond et al 2002?). In another study, investigators observed the Gpam-deficient mice were protected from diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC). In fact, while 13% of the GPAT1 +/+ mice were observed with HCC, HCC was observed in a mere 9% of the GPAT1 -/- mice (Ellis et al., 2012). Notably, when the two groups of mice were exposed to a tumor promoter, phenobarbital, and carcinogen diethylnitrosamine (DEN), the male GPAT1 -/- mice were observed to have 93% fewer visible liver nodules at 21 weeks, then 39% at 34 weeks, than the GPAT1 +/+ male mice. The GPAT1 -/- mice, due to their lower levels of GPAT1, were found to synthesize and store less TAG, instead, moving more towards beta-oxidation (Ellis et al., 2012). More recently investigators used a combination of diet and chemicals to induce increasing degrees of NASH and fibrosis in mice. They observed that mouse Gpam mRNA and protein levels directly correlated with increasing severity of NASH and fibrosis. Thus, all these data support the directionality of GPAM as disease-promoting and that down-regulating GPAM could be beneficial to patients with NASH.



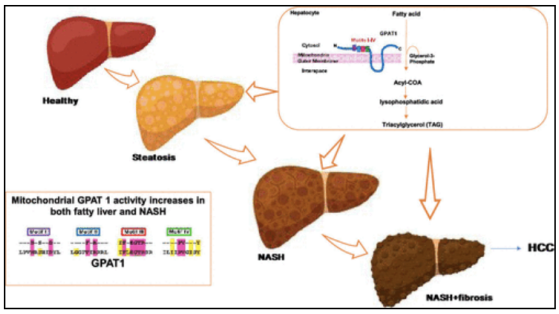
Lam *et al.,* 2015



Loh *et al.,* 2018

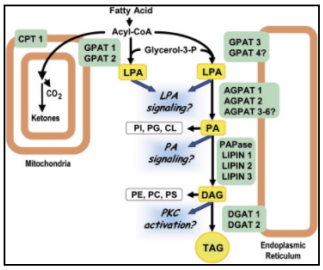
In the past, modulating a therapeutic target like GPAM, was challenging in that it was difficult to specifically regulate GPAM and not its closely related isoforms, which led to undesirable off-target effects. The use of RNA interference (RNAi) as a therapeutic modality allows for an innovative approach to specifically regulate formerly undruggable targets. One type of RNAi is small interfering RNA (siRNA). Over the last several years, scientists have figured out how to effectively deliver siRNA into hepatocytes and degrade mRNA. They do this by conjugating the siRNA complex to a very special kind of sugar molecule called GalNAC. GalNAc specifically binds to a receptor found only on hepatocytes, ASGR1. ASGR1 allows for internalization of the siRNA complex into the cell where it can then interact with RISC and degrade the target mRNA.

The studies presented in this report describe the first steps to prosecute *GPAT1* as a target for NAFLD. Specifically, the objectives were to validate a cell based model for evaluating Gpam mRNA expression, test the best conditions to knockdown Gpam mRNA expression using commercially available siRNAs to Gpam, and finally identify the best siRNAs to advance to in vivo proof of concept studies to determine efficacy of GPAM knockdown in a relevant rodent NASH model.



Liao *et al.*, 2020

Specifically, these studies evaluate whether siRNAs specific for *Gpam* are able to transfect a hepatocyte-derived cell line, Hepa1-6, and regulate *Gpam* mRNA expression. Top siRNA candidates should demonstrate robust, dose-dependent silencing of *Gpam* mRNA while not inducing any overt off-target effects, such as toxicity. These studies should provide the initial proof-of-concept rationale to support advancing the program to an in vivo context to demonstrate efficacy in a relevant rodent NAFLD model. Obtaining proof-of-concept for efficacy would provide justification to initiate a full human GPAM-specific siRNA screening and optimization campaign.



Loh *et al.*, 2018

Materials and Methods

In this experiment, *Gpam* mRNA expression was first confirmed in Hepa1-6 cells; the typical target expression being around 100 transcripts per cell under basic growth conditions. The Hepa1-6 cells are a hepatoma cell line derived from mice (*Mus musculus)*. For these studies, cells were cultured according to ATCC guidelines. Either 48 or 72 hours after siRNA transfection, the media was aspirated from the wells, the cells washed gently with dPBS, lysed using reagents provided in the RNeasy kit, and RNA was isolated from cell lysates. The RNA product was quantified and DNase treated prior to performing qPCRmeasure mRNA expression levels.

siRNA Invitro Experiment

siRNA was prepared by spinning the tube containing the lyophilized material, resuspending 5nmol in 100ul of 50uM NF-H2O and diluted 50uM stock into suspension buffer to a working concentration of 300nM. We began by diluting the siRNAs (the positive siRNA controls, Silencer Select GAPDH Positive Control siRNA and Silencer Select MALAT1 Positive Control siRNA, the negative siRNA controls, Silencer Select Negative Control No. 1 and No. 2 siRNA) to working concentration using Qiagen siRNA suspension buffer, then adding 4uL siRNA to

plate along with 16ul of plain media to siRNA. We then diluted RNAiMAX in plain media adding 20ul of that diluted RNAiMAX to siRNA and incubating it for 20 minutes at room temperature. We then added 80ul of cells in a complete media transfection complex, letting the plates sit 15-30 minutes at room temperature prior to incubating at 37C. For our DRAQ7 and Hoechst stain, we followed the live/dead imaging assay protocol, adding DRAQ7 and Hoechst to the lives cells, incubated for 30 minutes at 37C then imaged the plate live on Opera Phenix in ASF1-2125. We made up DRAQ7 and Hoechst in complete media, adding 10uL per well.

RNA Extraction

A Qiagen Rneasy kit was used to extract RNA from the harvested cells. First, an RLT Plus buffer was added to the cells and the mixture was vortexed for 30 seconds. The homogenized lysate was then centrifuged for 3 minutes. Next, lysates were transferred to the gDNA Eliminator spin column and centrifuged. The residual sample was then transferred to the RNeasy spin column, centrifuged, andthe flow through was discarded. This step was then repeated with a fresh buffer, and the resulting second flowthrough was again disposed. Lastly, the RNeasy spin column was placed into a new collection tube, water was added directly into the membrane, and centrifuged, resulting in eluted RNA.

Experiment 1: siRNA transfection optimization

The first experiment "performed" was to identify the best transfection condition.
When going into a person (or animal) we would use GalNAc-conjugated siRNA to deliver the siRNA to the hepatocyte, however, for cell assays, where we use the simplest duplex form of siRNA, a transfection reagent called RNAiMax is commonly used. But different cell types require different concentrations (low, med, high) of RNAiMax for good transfection. So, the first thing that needs to be done to develop the system is to identify the best concentration of RNAiMax to use. For this experiment, we used 2 different siRNA control reagents for this test: Malat1 and Gapdh. Both of these mRNAs are expressed at good levels in this cell type and there are validated siRNAs commercially available. Also, we used a single dose of 10nM and 2 controls were used: a vehicle-only condition & 2 commercial "negative" siRNAs (don't hit any target mRNA); for all of these we expect no change in expression (or very minor flux).
If transfection is successful, the expression of the target should be "knocked down"

These cells were incubated for 72 hours. At that point, the cells were washed, harvested, RNA was isolated and qPCR reactions were run looking for mRNA expression of Malat1 and Gapdh. Expression of a 3rd mRNA (TBP) was included. TBP is a housekeeping gene that should stay constant under all conditions and can be used to normalize the expression of the target mRNAs.

Experiment 1: siRNA transfection optimization

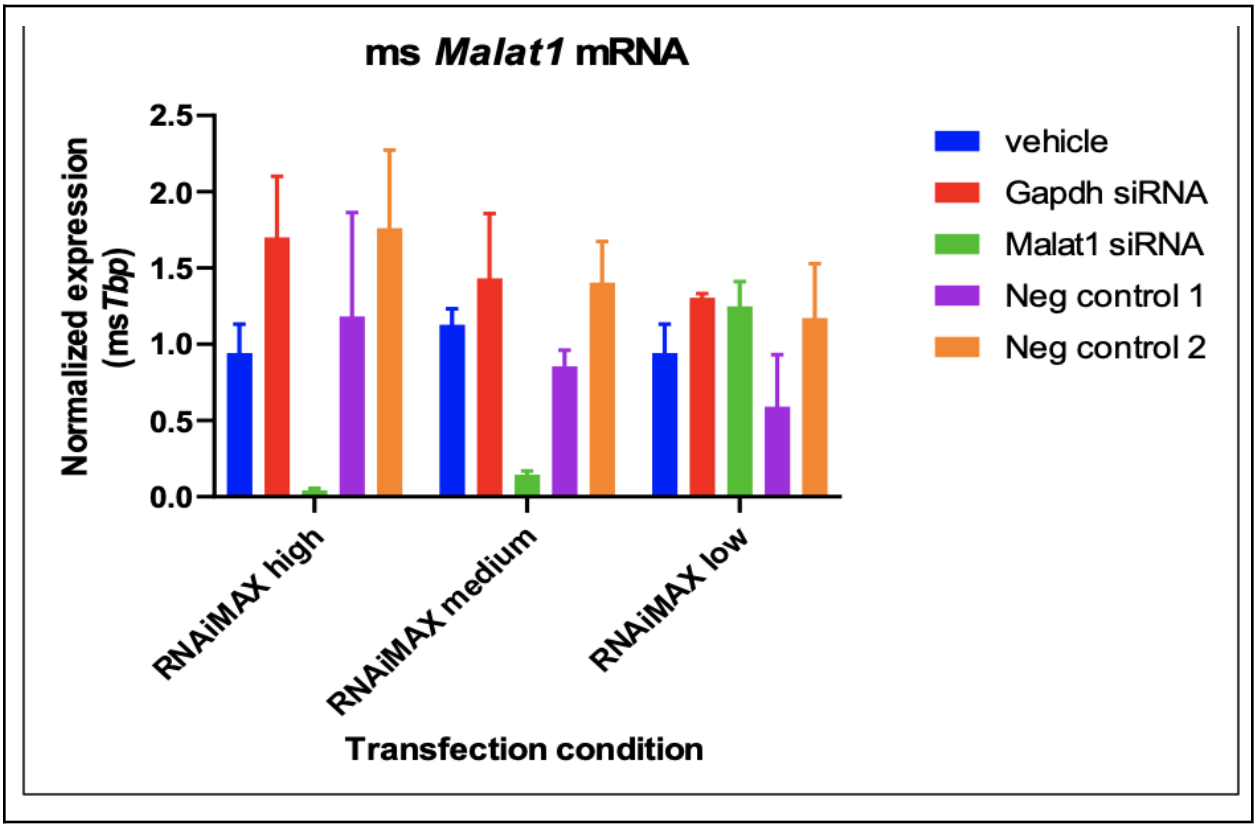


Figure 1

Malat 1 mRNA expression given different RNAiMAX concentrations at 72 hours given 10nM siRNA concentration per well. Data shown indicates the highest knockdown of Malat 1 siRNA with the highest concentration of RNAiMAX.

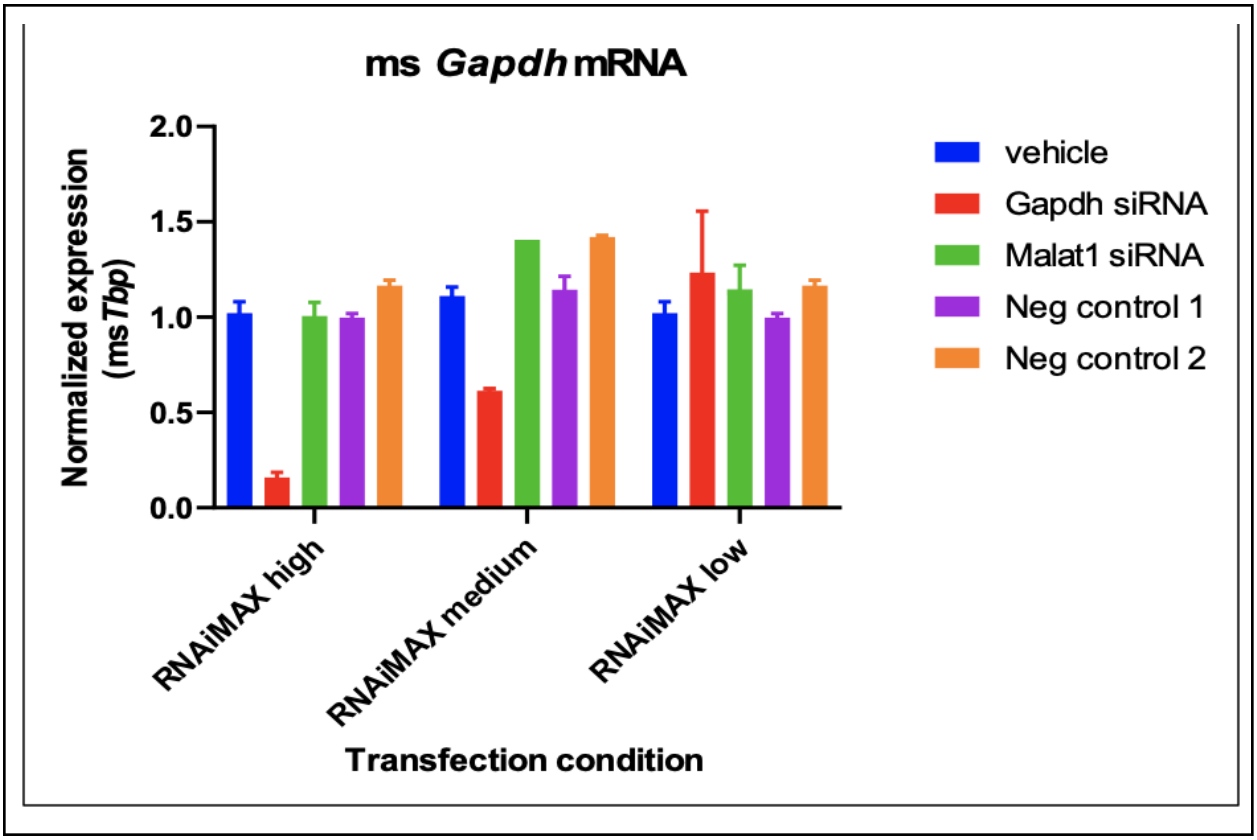


Figure 2

Gapdh 1 mRNA expression given different RNAiMAX concentrations at 72 hours given 10nM siRNA concentration per well. Data shown indicates the highest knockdown of Gapdh siRNA with the highest concentration of RNAiMAX.

Addition of vehicle leads to the "normal expression" level. We set this to 1. And then we calculate the relative silencing based off the vehicle condition. This will be the case for all the expression data I show. Malat1 mRNA was best silenced by Malat1 siRNA at the high condition of RNAiMax*; all others unchanged, and *Gapdh* mRNA was best silenced by Gapdh siRNA at the high condition of RNAiMax *; all others unchanged Thus, this experiment indicates that these hepatoma cells (hepatocyte cell line) need RNAiMax at "HIGH" for best transfection.

Experiment 2: Identification of time to maximal mRNA silencing

The next experiment was performed to identify the best time to achieve maximum knockdown. The experimental siRNAs were used at 10nM (the high dose) and with the "high" RNAiMax concentration. In this experiment, Gapdh siRNA was used as a positive control siRNA to assure strong knockdown under these conditions.

Experiment 2: Ideal time point for siRNA knockdown

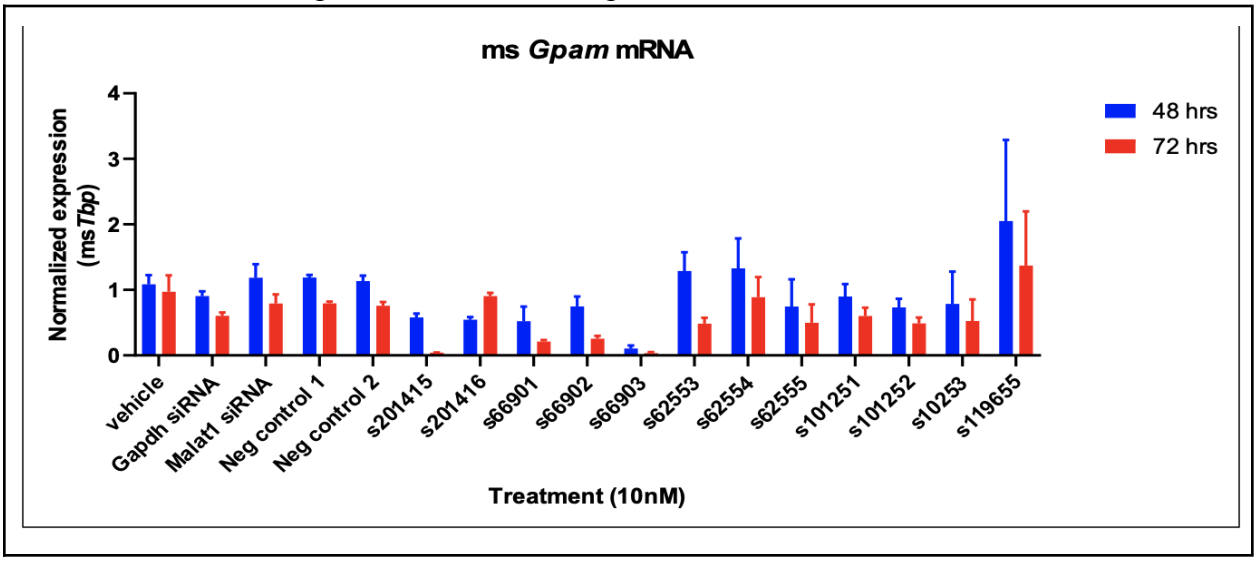


Figure 3

Determining the ideal time point for maximum siRNA knockdown with Gpam mRNA given 10nM siRNA concentration per well at 48 and 72 hours. Data shown indicates the highest knockdown at 72 hours. Notably, s201415, s66901, s66902, and s66903 look promising given these conditions.

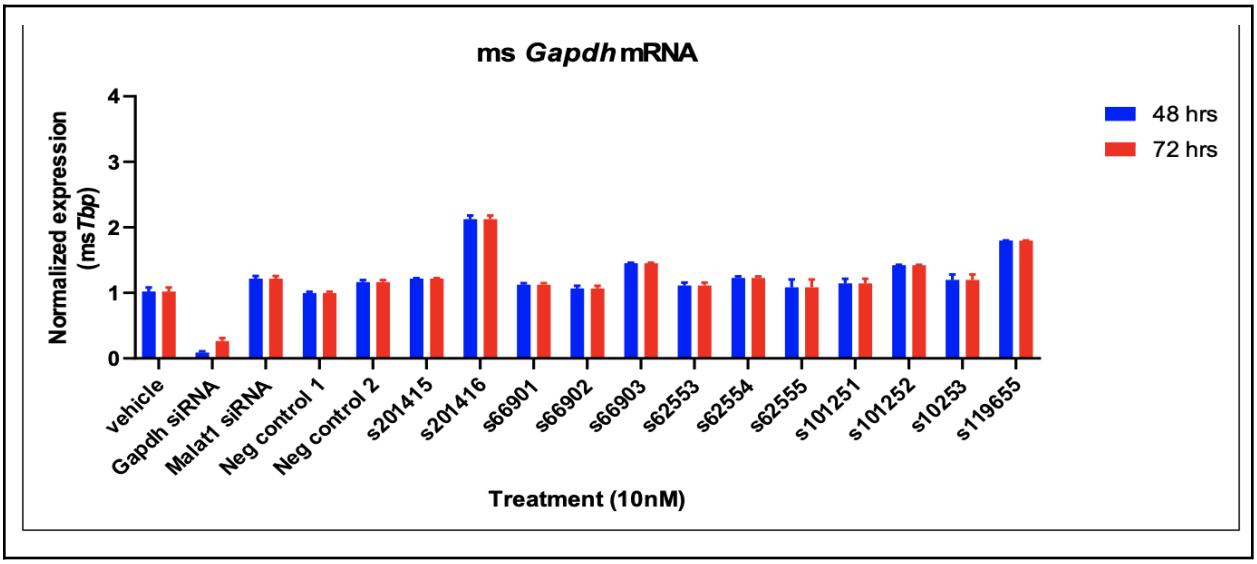


Figure 4

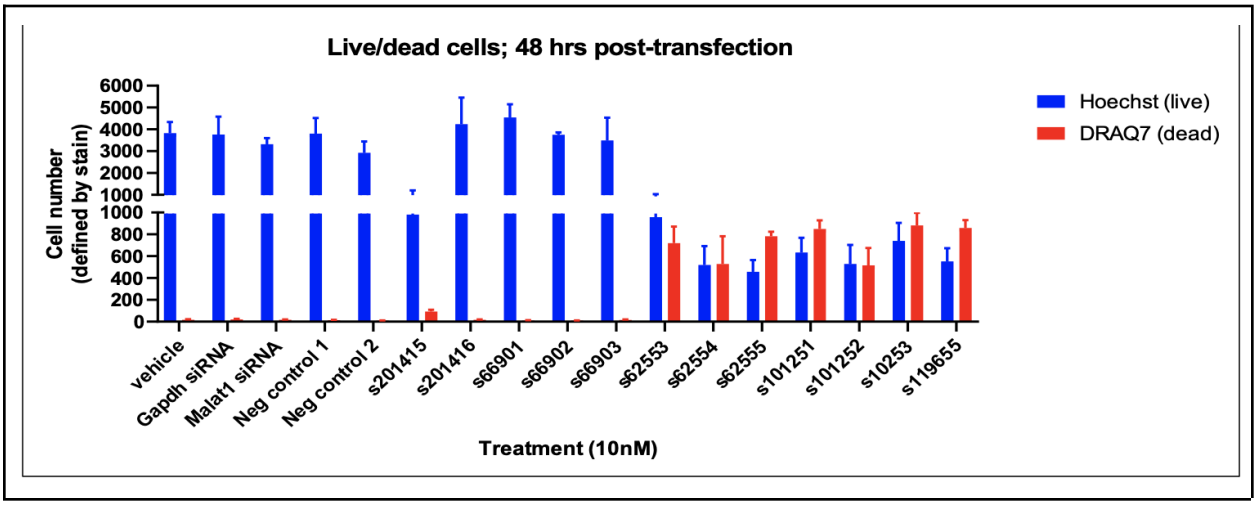
Determining the ideal time point for maximum siRNA knockdown with Gapdh mRNA given 10nM siRNA concentration per well at 48 and 72 hours. Data shown indicates that Gapdh has a much lower knockdown rate at both time points and for all candidates.

We see that several GPAM siRNAs worked well to knockdown Gpam mRNA expression; the 48 hr transfection is in blue and the 72 hr transfection is shown in red. Gpam knockdown was stronger after 72 hours of incubation. The Gapdh positive control efficiently knocked down Gapdh mRNA. For Gapdh, however, 48 hr was optimal under these conditions. From this, we can conclude that for the best conditions, we need to use high RNAiMax & incubate the cells for 72 hours to achieve maximum knockdown. Based on the results of this experiment we also determined that we wanted to focus on Gpam and targets s201415, s66901, s66902, and s66903 all have a lot of potential.

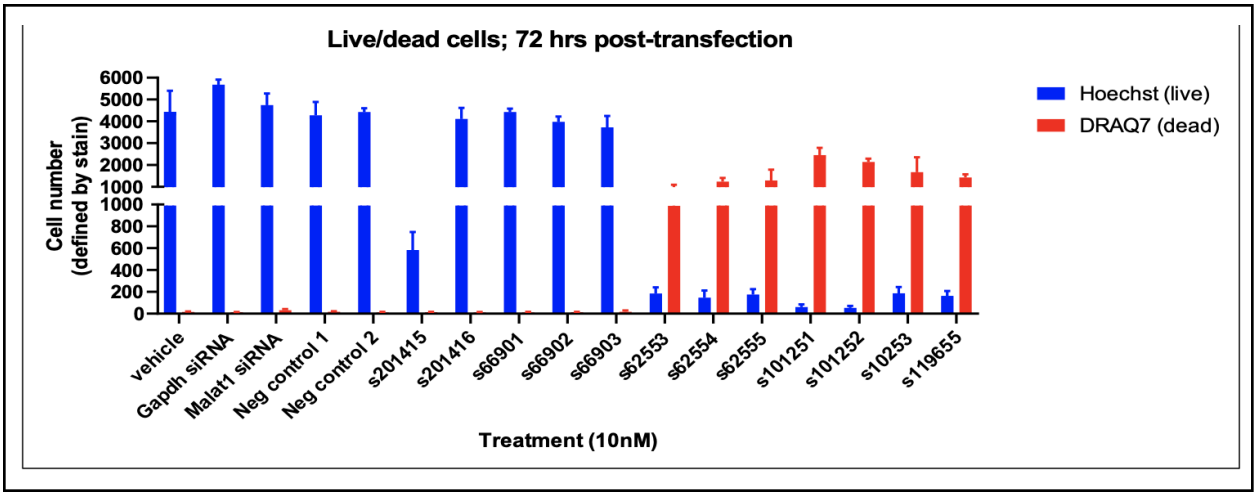
siRNA toxicity assessment

Finally, the last experiment we ran was to confirm that the candidate siRNAs for in vivo testing are not toxic to cells. This is an important checkpoint because we need to: Confirm knockdown is due to decreased on-target mRNA expression; not due to lost cells, and assure what is advanced to an animal is not toxic, or we may cause a bad event. To run this experiment, we again used the best knockdown conditions (RNAiMax "high" and 10nM of siRNA), to determine the number of liver cells versus dead cells over time, in this case, 48 and 72 hours. For this experiment we need to include a positive control for "cell death." So, to do this we used siRNAs to "essential" genes as positive controls. Because the target genes are "essential for life," if the transfection conditions are optimal, these siRNAs should induce cell death. The live/cell assay we used is to add special dyes to the cells at the time of harvest. The Hoechst dye stains healthy nuclei and the DRAQ7 dye stains dead cells. A few minutes after adding these dyes, a special imaging microscope platform can be used to scan the cell culture plate and record all the live and dead cells in each plate. Then imaging software is used to count the cells.

Experiment 2b: Transfection Efficiency



Determining the transfection efficiency for maximum siRNA knockdown with Gapdh mRNA given 10nM siRNA concentration per well at 48. Data shown indicates that at this time point, has a much lower knockdown rate though fewer dead cells.



Determining the transfection efficiency for maximum siRNA knockdown with Gapdh mRNA given 10nM siRNA concentration per well at 48. Data shown indicates that at this time point, has a much higher knockdown rate though more dead cells.

In this experiment our goal was to confirm that our siRNAs do not kill the cells at certain time points. Here, we tested the transfection efficiency at 48 and 72 hours using one concentration of siRNA, 10nm/well. We tested this using two positive siRNA controls, a mouse Gapdh and Malat, two negative siRNA controls, along with targets 1 through 5 and six essential genes to see how they react. The Hoechst dye stains healthy nuclei and the DRAQ7 dye stains dead cells. A few minutes after adding these dyes, a special imaging microscope platform can be used to scan the cell culture plate and record all the live and dead cells in each plate. Then imaging software is used to count the cells. Our results show that Gpam siRNAs are non-toxic to the cells, that s201415 is a bit toxic and that there are fewer live cells, especially at 72 hours, compared to the vehicle condition and negative controls. This confirms our assay conditions were good and all our controls and reagents work properly.

Experiment 3: Demonstration of dose-dependent knockdown of Gpam mRNA

The goal of this experiment was to demonstrate an "on-target" effect by showing increasing knockdown with increasing dose of siRNA. It is easy to select a siRNA that could hit something other than your intended target. There are several ways to confirm "on-target" activity. One way is a dose-response test. For the experimental siRNAs, again, we used the optimal conditions: "high" RNAiMax concentration & a 72 hr incubation.

Experiment 3: Ideal concentration for siRNA knockdown

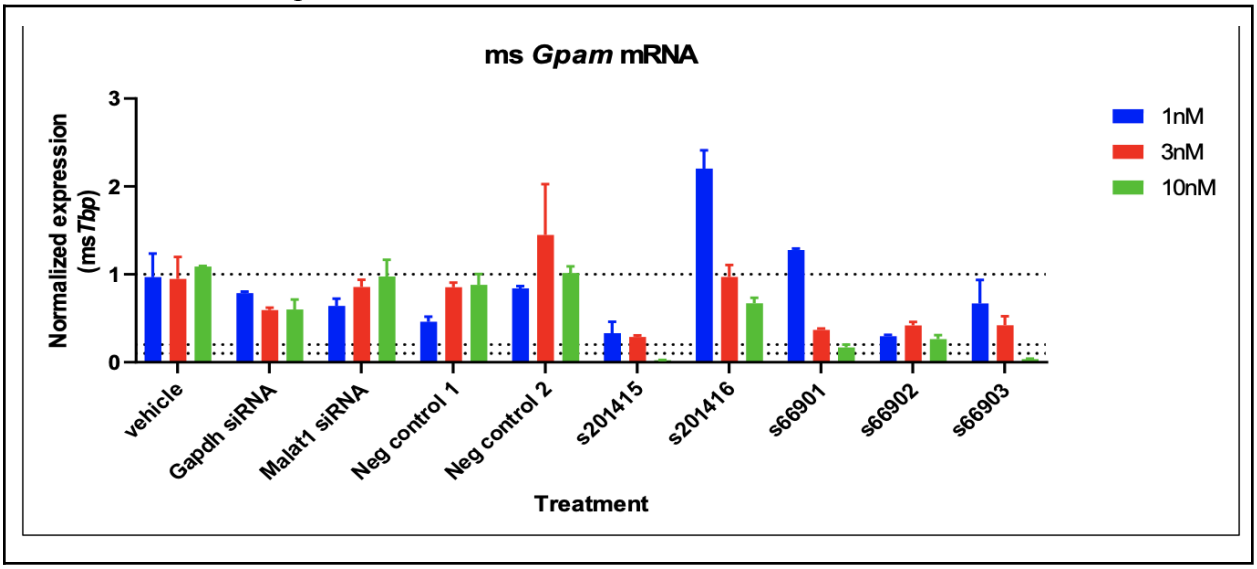


Figure 5

Determining the ideal concentration of siRNA per well for maximum siRNA knockdown with Gpam mRNA given a time point of 72 hours with concentrations of 1nm, 3nm, and 10nm per well. Data shown indicates the highest knockdown at 10nm. Notably, s201415, s66901, and s66903 look promising given these conditions.

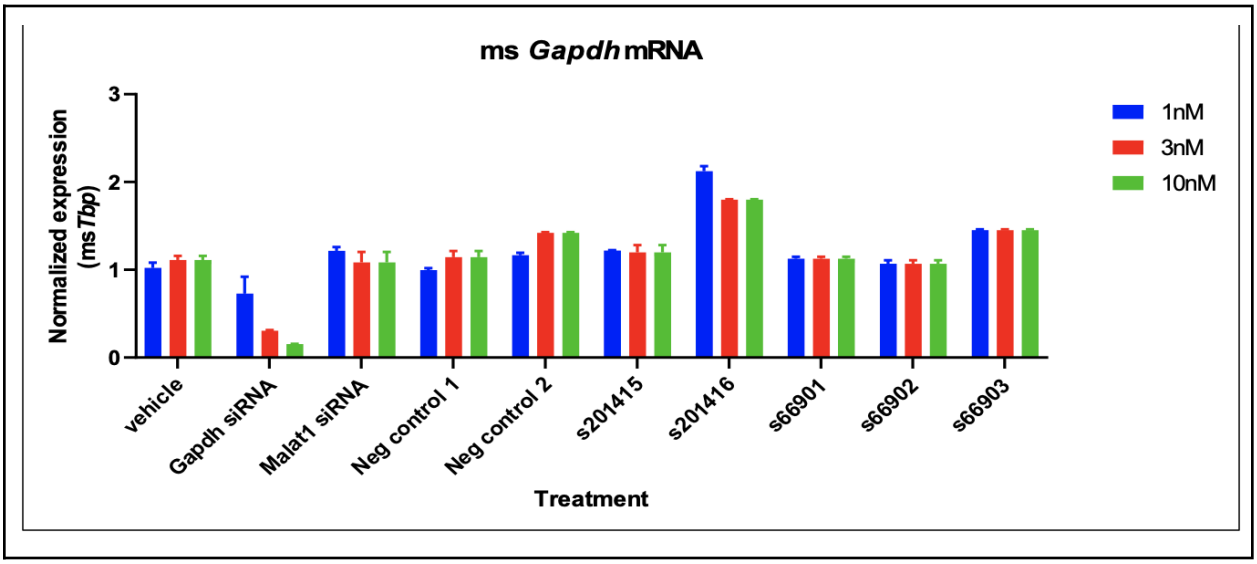


Figure 6

Determining the ideal concentration of siRNA per well for maximum siRNA knockdown with Gapdh mRNA given a time point of 72 hours with concentrations of 1nm, 3nm, and

10nm per well. Data shown indicates that Gapdh has a much lower knockdown rate at all concentrations and for all candidates.
This experiment demonstrates the "on-target" effect by showing increasing knockdown with increasing dose of siRNA. We tested this using two positive siRNA controls, a mouse Gapdh and Malat, two negative siRNA controls, along with targets 1 through 5 to see how they react. This experiment confirmed our suspicion that targets s201415, s66901, s66902, and s66903 all have a lot of potential based on the increase in knockdown along with the increase in siRNA concentration. Looking at all of our experiments, Gpam siRNA s66903 is a good candidate for advancing in vivo studies. It showed potency in the dose response and is not toxic to the cells.

Conclusions
The objective of this set of studies was to develop a therapeutic to knock down the expression of GPAM, our target. For this project, GPAM was identified as an attractive target, in particular because of the human genetic validation for its association with NAFLD that had been published. Furthermore, Mutations in GPAM associate with elevated NAFLD phenotypes, the known biology suggests knocking down GPAM would be beneficial in NAFLD patients, and because GPAM is expressed in hepatocytes, siRNA to GPAM may be a feasible therapeutic modality.

In designing our therapeutic we decided to use siRNA technology. It is challenging to make specific small molecule inhibitors but selectively targeting siRNAs to diseased cells or tissues will increase the silencing potency at the site of interest and thus make the treatment more effective. We can use GalNAC to direct to the hepatocyte, the diseased cells and sources of the issues, and knockdown the GPAM expression in the hepatocyte, hopefully stalling the progression of NAFLD if not reversing it entirely. This siRNA therapy is also the most likely to be accepted and improved by the body as diseases such as NAFLD are currently incurable by conventional drugs. And though this technology is unlikely to be able to reverse cirrhosis, it shows the potential to have the ability to at least stop its progression.

In the context of siRNA therapeutics, from these experiments we see new possible means to target a genetic driver, a novel way to regulate NASH and potentially bring treatment to the growing millions that suffer from NASH. As there are no other NASH therapeutics that have been improved, this one brings a lot of potential as it's targeting a protein that comes with human genetic validation making it more likely that the body will respond well and as we predict it should compared to the other therapeutics in the works. The data we collected means that our therapeutic looks very promising. We have determined the proper time points, concentrations, and targets for our therapy. We have yet to do a functional study regarding GPAM and its regulation of triglyceride synthesis but it appears that we should be able to almost completely knock down GPAM expression and thus more properly regulate triglyceride synthesis in the liver with the development of this therapeutic. For this, our next step would be to run an in vivo POC study to demonstrate siRNA-mediated regulation of GPAM protein expression can prevent

NASH progression in a mouse model of NASH. If successful, a large-scale screening and optimization campaign would begin to generate new human specific- GPAM siRNA-GalNAc conjugates to develop for the clinic.

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